

RADIOSENSITIZING GLIOBLASTOMA IN A RAT MODEL USING L-BUTHIONINE-SR- SULFOXIMINE (BSO)

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ABSTRACT

Glioblastoma multiforme (GBM) is the most aggressive and most common primary brain tumor in adults accounting for 50-60% of primary brain tumors. The prognosis for patients with GBM remains poor and treatment is mainly palliative with a mean survival time of less than one year. Radiotherapy is used extensively in the management of glioblastoma either alone or in combination with surgery and/or chemotherapy. However, this tumor is one of the most resistant tumors to radiotherapy thus limiting the benefit of this form of treatment.

Studies have shown that malignant tumors have a high content of glutathione an antioxidant responsible for protecting the cells against damage from free radicals (mainly superoxide, hydroxyl and hydrogen peroxide). It is well established that glutathione, by neutralizing these free radicals plays a major role in radioresistance. Glioblastoma has relatively high levels of glutathione. In this study, by reducing the glutathione content of glioblastoma in a rat model, we were able to investigate the effect of this reduction in enhancing the effect of radiotherapy as a form of treatment for glioblastoma multiforme in a rat model.

By injecting L-Buthionine-SR-Sulfoximine (BSO) in to the tumor tissue, the glutathione content of the tumor was reduced by about 70% of its initial value. When administered into the tumors 2 hours prior to radiotherapy the animals so treated had a significantly longer median survival time compared with animals that received radiotherapy alone.

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DEDICATION

To my mother Nima Abdelraheem and my father Ali Ataelmannan
Who inspired and supported me in every step of my pursuit of knowledge.

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LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BCNU	1,3-Bis(2-Chloroethyl)-1-Nitrosourea
BSO	L-Buthionine- SR-Sulfoximine
CCDP	cis-Dichlorodiammineplatinum (II)
CED	Convection Enhanced Delivery
DEM	Diethyl Maleate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
GBM	Glioblastoma Multiforme
GPX	Glutathione Peroxidase
GSH	L-glutamyl-L-cysteinylglycine
GSSG	Oxidized-Glutathione
H & E	Hematoxylin and Eosin
HPLC	High-performance liquid chromatography
H ₂ O ₂	Hydrogen Peroxide
MRI	Magnetic Resonance Imaging
PBS	Phosphate Buffered Saline
ROS	Reactive Oxygen Species
RT	Radiotherapy
SOD	Superoxide Dismutase

HYPOTHESIS

Reducing the cellular content of tumor glutathione in a rat glioma model using L-Buthionine-SR-Sulfoximine (BSO) will enhance the effect of radiotherapy and improve survival outcome following treatment.

1. Introduction

Brain tumors are the most common solid tumors in children and the second most common malignancy of childhood (1). In North America and Western Europe there are about 6-11 new cases of primary intracranial tumors per 100,000 per year in men and 4-11 new cases in women (2, 3). Asian populations have a lower incidence rate than Europe and North America (3). The incidence in developing countries is lower but this may be due to under reporting (4). However, Caucasians tend to have a higher incidence when compared to people of African or Asian origin (5, 6) and thus there may be ethnic differences in susceptibility to brain tumors. The incidence of glioblastoma and germ-cell tumors is 3.5 times more frequent in Caucasians than in African Americans (7).

Some studies have shown an increase of about 1-2% per year in the incidence of brain tumors during the 1980s and 1990s (8) both in the elderly (9) and in children (10). However this increase may be due to introduction of better diagnostic techniques which have greatly improved diagnosis of neurological diseases (11). In some of the Scandinavian countries, increased incidence was seen in the late 1970s and early 1980s a period which coincided with introduction of improved diagnostic methods (12). After the 1980s the incidence for both sexes remained relatively stable (12).

In North America and Western Europe the mortality rates from nervous system tumors are approximately 4–7 per 100,000 person per year in men and 3–5 in women (3, 13). The mortality and incidence rates in both sexes show similar patterns worldwide. Changes in ratio between incidence and mortality in different regions usually reflect differences in the success of disease management (13). The survival of glioma patients (with exception of pilocytic astrocytoma) is still very poor (14). For glioblastoma patients, the 5-year survival rate is less than 3% (15, 16). Older age at diagnosis is a poor prognostic factor for both sexes (16).

Primary glioblastoma arise de novo and account for 60% of cases in adults 50 years of age and older (17). They usually manifest clinically in less than 3 months. Secondary glioblastoma account for the remainder and are typically seen in younger patients, usually less than 45 years. They develop through malignant progression from a low-grade astrocytoma (grade II or III). The time required for this progression ranges from 1 to 10 years, with a mean interval of 4-5 years.

Primary brain tumors are classified according to the tissue from which they arise. The most common are gliomas which arise from tissue that supports and nourishes the brain, the glial tissue. They account for about 45-50% of all primary brain tumors and include astrocytomas, oligodendrogliomas, and tumors with mixtures of two or more of these cell types (17). The microscopic appearance of brain tumors is used to grade brain tumors from I to IV to indicate their level of malignancy with grade IV being the most malignant with a tendency to invade normal surrounding brain tissue and a potential for local spread. These are known as glioblastoma multiforme and account approximately for 50% of astrocytomas. Grade III astrocytomas grow more rapidly than lower grade tumors and tend to invade normal surrounding brain tissue. Grades I & II astrocytomas account for 25-30% of all gliomas and are the least malignant of the astrocytomas although they do have the potential to progress to higher grade astrocytomas.

Glioblastoma primarily affect adults and they are located preferentially in the subcortical white matter of the cerebral hemispheres. They may affect the brain stem in children and the spinal cord, but this is much less common. These tumors are composed of poorly differentiated astrocyte cells with a high mitotic index and a necrotic centre. To maintain their rapid growth, the tumors induce new blood vessel formation. These tumors may develop from lower-grade astrocytomas (grade II) or anaplastic astrocytomas (grade III) but more commonly arise de novo without any evidence of an antecedent lesion (17).

The treatment of GBM remains a great challenge with current therapies being mainly palliative and no significant advances during the last two decades (18, 19). Without therapy patients usually die within three months while patients treated with optimal therapy have a median survival of approximately 1 year (20). However, a recent study using radiotherapy plus concomitant and adjuvant Temozolomide showed an increase in the median survival of patients to 14.6 months as compared to 12.1 months when using radiotherapy alone after a follow up of 28 months (21).

One of the great difficulties in treating GBM arises from the fact that the tumor cells have an infiltrative nature with no clear border around them. Cancer cells may spread throughout the brain and further away from the site of the bulk tumor. Even in

cases where radical surgery can be performed it is impossible to get a clear margin with complete eradication of the tumor (19).

The treatment of GBM involves a combination of surgery, radiotherapy and chemotherapy. Surgery helps to alleviate some of the patient's symptoms by decreasing the raised intracranial pressure. It also aims to remove all tumor tissue without causing neurological deficit. However, this is usually not feasible and may be compromised by the location of the tumor as well as its infiltrative nature. Overall, the survival of patients with GBM who undergo surgery as the only form of treatment is on average approximately 14 weeks (17, 22).

Radiotherapy after surgery helps prolong the survival of patients. Even when it appears that the entire tumor has been surgically removed, microscopic cancer cells remain in the surrounding brain tissue or at distant sites from the bulk tumor. Radiation targets the residual tumor with the goal of reducing its size or stopping its progression. If the entire tumor cannot be removed safely, postoperative radiotherapy is often recommended. Radiotherapy is also used instead of surgery for inaccessible GBM. The initial approach to treating gliomas with radiotherapy was whole-head irradiation. This was later abandoned due to the substantial neurological deficits that resulted, sometimes appearing a considerable time after treatment. Current clinical practice uses a more focused radiation field that includes only 2-3 cm beyond the periphery of the tumor site.

In many instances, radiotherapy can induce a phase remission often marked with stability or regression of neurological deficits as well as a reduction in size of the tumor. However, the response of GBM to radiotherapy varies. Combining radiotherapy with surgery results in a median survival of 40 weeks (17, 22). Radiation therapy therefore prolongs survival and improves quality of life over the intermediate period of time. This is followed by a further clinical deterioration and eventually leads to the demise of the patient. In recurrent GBM the mortality rate approaches 100% within a few weeks to months. Following the diagnosis of recurrence, a reasonable quality of life is usually limited to 10 weeks in these group of patients (23).

The use of brachytherapy (a form of radiotherapy in which a radioactive source is placed in or near the tumor) or radiosurgery (a form of non-invasive brain surgery in

which focused radiation beams are delivered to a specific area of the brain to be treated) in treatment of gliomas has been one of the major advances in the treatment of these tumors using radiation therapy. Median survival time for patients receiving brachytherapy has been 18-20 months (24). Unfortunately, due to restrictions because of tumor size and location, only 25-40% of patients are eligible to receive this form of treatment. Those who receive the treatment had a median survival of 16 months as compared to 6 months for those who did not. However, selection bias was thought to be the reason for this apparent benefit of brachytherapy (25). Phase III clinical trials that used random assignment of patients who met the inclusion criteria for brachytherapy versus a control failed to show a statistically significant benefit of brachytherapy, although there was a numerical advantage for patients receiving brachytherapy (26, 27).

A popular alternative to brachytherapy is radiosurgery using either a linear accelerator or the Gamma Knife. Radiosurgery, unlike brachytherapy which requires surgery and 5-7 days of treatment sessions, does not require surgery and can be conducted in a single treatment session. It is also associated with a lower rate of radiation necrosis. However, some studies have indicated that there is no difference in benefit between the two forms of treatment, at least for recurrent GBM (28). Some centers use fractionated radiosurgery for the treatment of larger tumors. This consists of one radiation session per week for four weeks. However, different studies have shown this form of treatment to have conflicting results when compared to single radiation sessions ranging from being similar to very positive (29, 30).

Chemotherapy has been of slight benefit in increasing survival of patients with GBM (31). Chemotherapy may be given before, during or after radiation therapy. It is often administered at the time of tumor recurrence. The response rate to most of the chemotherapeutic agents used is no greater than 30-40% and most fall into the range of 10-20% (32). Patients receiving RT alone have a two-year survival rate of 0-10% as compared to a survival rate of 15-25% for patients receiving a combination of radiotherapy and chemotherapy (33).

Radiation therapy is still used extensively in the management of glioblastoma. However, one of the major limitations of this form of treatment, is the intrinsic or

acquired resistance of this tumor to radiation therapy whether used alone or in combination with surgery (34). Exposure of cells to ionizing radiation causes the formation of reactive oxygen species (ROS), mainly superoxide, hydroxyl radical and hydrogen peroxide inside the cells. This is thought to be one of the main mechanisms by which ionizing radiation causes cell damage and death. Antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) can be used to eliminate ROS in cells. This diminishes the action of ionizing radiation and protects the cell against cell damage. It is also known that these antioxidant enzymes play a role in radioresistance and chemoresistance. Glioblastoma which is one of the most radioresistant tumors has relatively high levels of these enzymes (35).

2. Literature Review

2.1 Glutathione

Glutathione (L-glutamyl-L-cysteinylglycine; GSH) is a tri-peptide composed of three amino acids: glutamic acid, cysteine and glycine. Glutathione is essential to all life and is found in tissues of virtually all plants and animals at an intracellular concentration of 1–10 mM in mammalian cells (36). In 1888 while working with yeast cells de Rey-Pailhade found out that yeast cells contain a substance that is responsible for forming hydrogen sulfide when the cells were crushed with elemental sulfur. He also found the same substance to be present in a number of plant and animal tissue including beef liver, sheep brain, fish muscle, egg white and freshly picked asparagus. de Rey-Pailhade named this substance philothion (from the Greek “sulfur loving”). In 1921 Hopkins renamed philothion as glutathione (37). It has been estimated that more than 90% of the nonprotein sulfur of cells is in the form of glutathione (38).

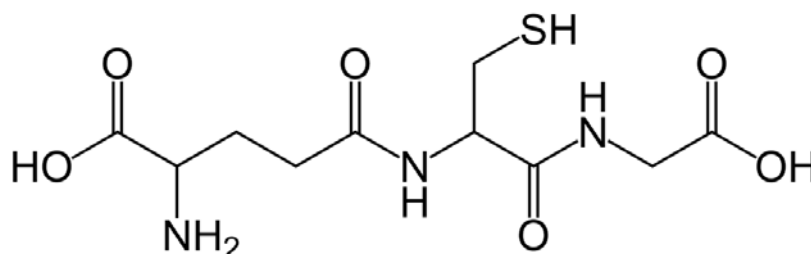


Fig 2.1: The structure of glutathione

2.2 Biosynthesis and Degradation

GSH is synthesized from L-glutamate, L-cysteine, and L-glycine by the consecutive action of γ -glutamylcysteine synthetase and GSH synthetase in two ATP (or energy requiring) dependent reactions (39). The first reaction is the rate-limiting step and is effectively inhibited by GSH feedback. However, when GSH is oxidized, feedback inhibition is lost and the availability of L-cysteine as a precursor can become the rate-limiting factor (40). Once GSH is produced, it will either function by itself or be degraded to participate in other metabolic pathways such as the γ -glutamyl cycle

that can supply amino acid precursors for GSH synthesis (39). The enzymes involved in GSH degradation are γ -glutamyl transpeptidase and dipeptidases.

Under normal conditions, the majority of glutathione exists in reduced form (GSH). Oxidation of the GSH to form oxidized-glutathione (GSSG) is carried out either by direct interaction with free radicals or more often when GSH acts as a cofactor for antioxidant enzymes such as GSH peroxidases during the reduction of H_2O_2 (both selenium-dependent and non-selenium-dependent forms) and phospholipid hydroperoxide GSH peroxidases. The ratio of the GSSG/GSH couple can serve as an important indicator of the cellular redox environment.

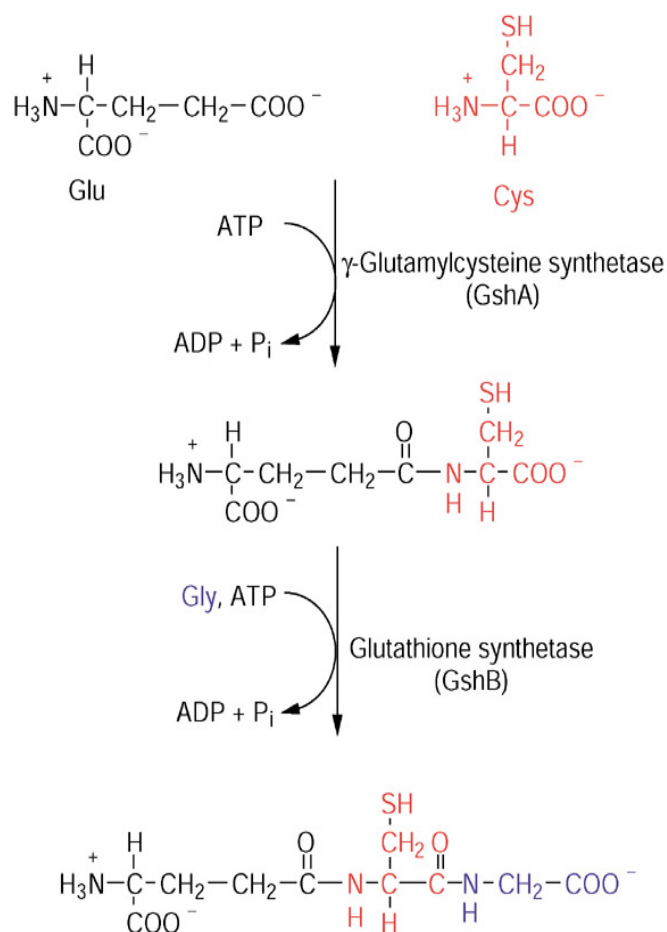


Fig 2.2: Pathway for glutathione biosynthesis.

2.3 Biological Functions of Glutathione

Glutathione has a number of cellular functions. It is an effective intracellular reducing agent. It functions in catalysis, metabolism, and transport as well as protection of cells against foreign compounds, free radicals and reactive oxygen compounds. GSH is also an active participant in reactions that destroy hydrogen peroxide and organic peroxides. It also protects DNA from free-radical damage.

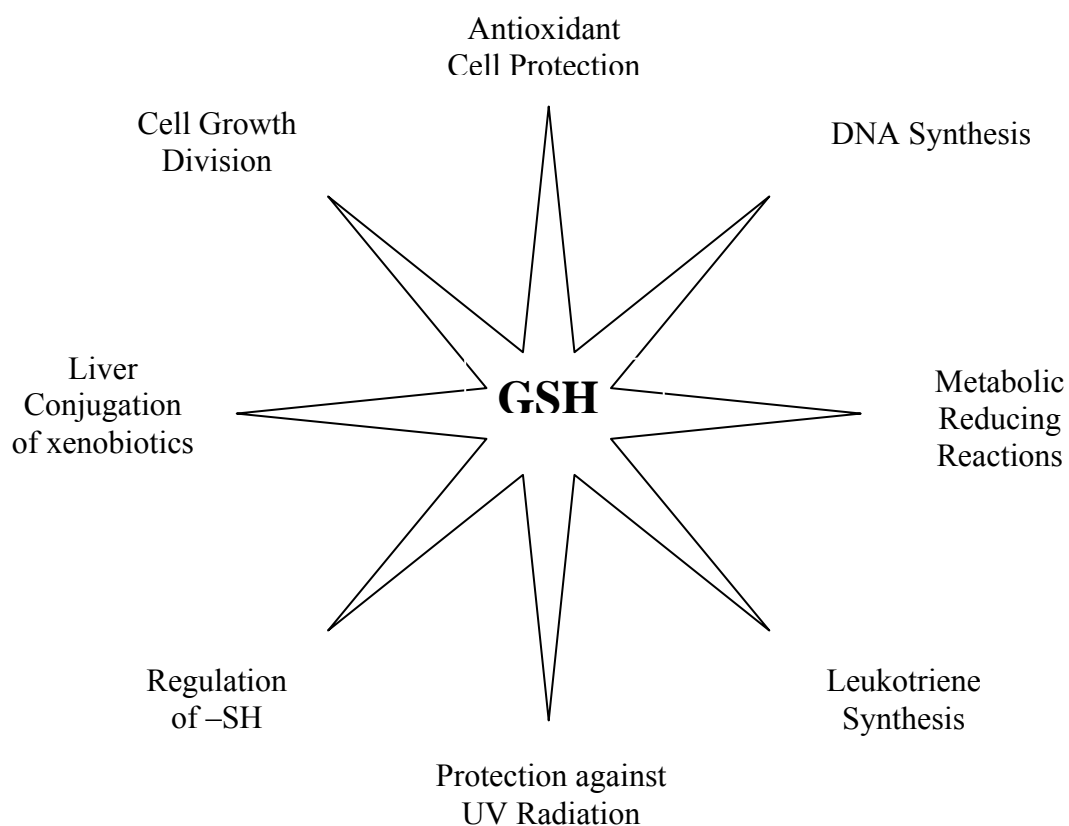


Fig 2.3: Functions of glutathione.

2.4 Antioxidant Activities

Aerobic metabolism in aerobic organisms leads to oxygen radical stress as a byproduct. This results in the formation of highly active intermediates, namely hydrogen peroxide and superoxide which promote further oxygen radical production that lead to cellular damage (41). Being the major endogenous soluble antioxidant in mammalian cells, GSH provides protection against this oxidative stress. It functions as a substrate for the antioxidant enzymes GSH peroxidase and phospholipid hydroperoxide GSH peroxidase that convert peroxides into less harmful alcohols, water and GSSG (42). GSH can directly scavenge radicals and peroxides via mixed disulfide formation or upon oxidization to GSSG and thus protect the cells against oxidative stress by a non-enzymatic action. This prevents the harmful effects on tissues associated with peroxidation of cell membrane lipids (43). There is evidence that a variety of oxygen radical stresses can result in depletion of GSH and formation of GSSG the short term (44).

2.5 Modulation of the Immune System

GSH is required in the normal function of the immune system and production of an immune response. Intracellular GSH has been shown to modulate T-cell function, including the binding, internalization, and degradation of interleukin-2 (45). It is also important for DNA synthesis (46). GSH can also enhance cytotoxic T-cell activation, proliferation and differentiation (47). Studies have shown not only that in vivo administration of GSH can activate cytotoxic T-cells, but also that depletion of intracellular GSH can inhibit the activation of lymphocytes, suppressing their cytotoxic functions and increasing susceptibility of the cell to radiation damage (48).

2.6 Detoxification

Mammalian cells are involved in many metabolic activities and are thus exposed to many chemical toxins such as heavy metals and drug metabolites. They are also exposed to physical damage caused by environmental pollutants, smoke and ultraviolet radiation. All these physical and chemical toxins lead to cellular damage, and if left unchecked can eventually lead to cellular death. There are cellular

mechanisms which are responsible for detoxification and counteracting these damaging effects. These mechanisms include phase I reactions (which can lead to either to activation or inactivation of a drug), hydrolysis or reduction and phase II reactions which involve the conjugation of toxins to endogenous chemicals with little or no chemical activity. A major phase II reaction in mammalian cells is conjugation to GSH. This is particularly important in the detoxification of electrophilic substances such as epoxides, alkenes, halides and heavy metals (49). Organs that have the highest exposure to toxins such as the lungs, liver and kidneys are the richest in GSH. Elimination of GSH conjugates takes place via an ATP-dependent glutathione S-conjugate export pump called GS-X pump (50).

2.7 Glutathione and Cancer

GSH has a complex role in both protection of cells against cancer and cancer therapy. While GSH plays an important role in the detoxification of carcinogens and protection of cells against cancer development, it is elevated in many types of tumors where it is thought to play an important role in increased resistance of these tumors to chemo- and radiotherapy (51-54). Such elevated levels are seen in many cancer tissues including breast, lung, colon, ovary, liver and larynx (55-63).

2.8 Glutathione and Cancer Therapy

Free radicals can cause cell injury and other pathologies including cancer if they are not neutralized. Intracellular GSH helps to counteract the effect of these free radicals thereby protecting the cells against potential damage. While increasing GSH levels within normal cells may help protect the cells against damage and development of cancer due to free radicals, many studies have employed various methods to reduce GSH in cancer cells in an attempt to make them more susceptible to chemotherapy and radiotherapy. Some studies have shown that by using BSO to reduce the GSH in cell culture lines, different responses to chemotherapy in normal versus tumor cells with the tumor cells lines being more susceptible (64, 65).

2.9 Chemical Depletion of Glutathione

Tissue glutathione levels are intentionally altered for various experimental studies. Different cell populations in a given organ may not necessarily have the same GSH concentration and there may be discrete subcellular pools of GSH (66, 67). Studies have shown that depletion of tissue glutathione by more than 70% of normal values can lead to changes in xenobiotic metabolism (deactivation and secretion of foreign chemicals such as drugs which happens mostly in the liver) and increased toxicity of electrophilic metabolites. Depletion of hepatic GSH in rats resulted in increased toxicity of acetaminophen (68) with similar effects observed in mice (68), goats (69) and hamsters (70).

Different methods are used to lower GSH levels. One of the methods used is the administration of compounds that react enzymatically with GSH to form conjugates. Depletion of GSH can also be achieved by inhibition of GSH synthesis in organs with a high turnover rate. Another method for GSH depletion is conversion of GSH to its oxidized form, GSSG. However, the choice of depleting agent depends on what system is being studied e.g. in the liver where glutathione transferase levels are high, compounds whose reaction with GSH is enzyme-catalyzed will be more effective. For the same reason, such compounds are more effective in rodents than rabbits or monkeys with lower transferase concentration (71). In the kidneys which have a high GSH turnover rate, inhibitors of GSH synthesis are most effective (72) while in organs or cells with high concentrations of microsomal monooxygenases, depleting agents requiring activation by these enzymes are the most effective.

When choosing a depleting agent, other than the efficacy of the agent in depleting GSH, its side effects must also be taken into consideration. Many GSH depleting agents can result in lipid peroxidation and cell lysis in isolated liver cells (73) and in animals so treated, an increased rate of lipid peroxidation was observed with liver homogenates probably resulting from GSH depletion (74).

Tissue GSH levels are lowered by many electrophilic chemicals by reacting with the sulfhydryl group of GSH. By covalently binding nonselectively to nucleophiles, some highly electrophilic compounds can cause toxic effects. This

makes compounds that react moderately and which require enzymatic catalysis to produce GSH depletion a much better choice.

2.10 Types of GSH Depleting Agents

Based on their mechanism of action substances that bring about glutathione depletion are divided into different groups:

2.10.1 Substrates of Glutathione Transferases

These include α,β -unsaturated carbonyl compounds which are weak electrophiles. In the presence of glutathione transferases they react with GSH to bring about its depletion (75). Diethyl maleate (DEM) is the most widely used compound in this group (76). Administration of DEM intraperitoneal reduces hepatic GSH levels in rats to less than 20% of control values in 30 minutes with its effect lasting for 2 to 4 hours (74, 75, 77, 78). The GSH concentrations in the rat red blood cells, kidney, lung and brain are also depleted though to a lesser extent than in the liver (79). GSH levels in liver, kidney and skin were reduced by repeated topical application of DEM to mice (80). DEM has also been used invitro to remove GSH from rat liver and isolated rat hepatocytes (68, 81). In addition to GSH depletion, DEM produced a other undesirable effects such as increased bile flow in rats and dogs (77) and increased hepatic microsomal heme oxygenase activity in rats (82).

Phorone (diisopropylidene) is another α,β -unsaturated carbonyl compound that can be used to produce GSH depletion (83). Phorone when given to rats intraperitoneally resulted in depletion of hepatic GSH to less than 10% of control levels in 2 hours. However it may cause hepatotoxicity. Acrylonitrile (76), acrylamide (79), and esters of acrylic acid (76) are also other unsaturated compounds that can deplete GSH by enzyme-catalyzed reactions. Other compounds that can cause GSH depletion by enzyme-catalyzed reactions include aliphatic halo compounds such as iodomethane (84), aromatic halo compounds such as 1-chloro-2,4-dinitrobenzene (85), epoxides such as styrene oxide (86) and organophosphates such as the anticancer drug BCNU (carmustine) (87).

By metabolism to reactive electrophiles, some relatively inert compounds can cause tissue depletion of GSH. Acetaminophen (88), bromobenzene (89), fluoroxene (90) and doxorubicin are examples of such compounds. However, these compounds can cause hepatotoxicity if excess levels remain after GSH has been depleted.

2.10.2 Oxidants

Compounds that oxidize GSH to GSSG have been used mainly to deplete GSH in *in vitro* (Latin: within the glass) cell preparations. The main agents in this class are diazenecarboxylic acid derivatives such as diamide (91). One of such compounds, sodium tetrathionate has been used to deplete GSH *in vivo*, resulting in diminished kidney, erythrocyte and liver GSH levels in rats to values ranging between 15 to 60% of control levels in 2 to 5 hours (79); however, these compounds are nephrotoxic.

2.10.3 Inhibitors of Biosynthesis

Intracellular glutathione is normally maintained within a certain range that may vary from one cell or tissue type to another. In this state of equilibrium, GSH biosynthesis is balanced by rate of its utilization. Increasing the rate of GSH utilization or decreasing its biosynthesis will result in decreased GSH levels. Methionine sulfoximine inhibits two enzyme systems - glutamine synthetase and γ -glutamylcysteine synthetase (92). Thus both glutamine and GSH levels were depleted when mice were treated with methionine sulfoximine. This resulted in both convulsions and death in the treated mice (92, 93). In search for a more specific inhibitor of glutamylcysteine synthetase, different methionine sulfoximine analogs were synthesized and evaluated both *in vitro* and *in vivo*. The most specific inhibitor of glutamylcysteine synthetase was found to be L-buthionine sulfoximine (BSO) (94). Furthermore, unlike its higher analogs such as hexathionine and heptathionine which are quite toxic, BSO was found to have no toxic effects even when administered in very high concentrations (92, 94, 95). The toxic effects of the higher analogs is thought to be due to non-specific membrane damage or due to insolubility of these compounds at physiologic pH (92).

2.10.4 Miscellaneous Compounds

There are a number of compounds whose mechanism of action is unknown and which do not fit into any of the above groups but can also cause depletion of GSH in cells or tissues. Ethylmorphine is one of such compounds. It causes depletion of GSH in isolated rat hepatocytes (73). Aspirin also caused reduction of rat hepatic GSH levels to about 70% of control levels in 4 hours when given intraperitoneal. (96). Fasting also leads to depletion of hepatic GSH. When fasted for 18 hours rats had significantly lower hepatic GSH levels than fed controls. However, GSH levels in the kidney, brain and erythrocytes were not significantly affected (79).

2.11 Choice of Depleting Agent

The choice of a chemical for lowering GSH has to take into account the efficacy of the compound as well as its unwanted side effects. The site of action of the depleting agent is also very important as not all depleting agents cause GSH depletion in all tissues. Substrates of glutathione transferases can only act where these enzymes are present such as the liver and kidney. DEM is a powerful compound for depleting hepatic GSH in vivo and has few undesirable effects. Also α,β -unsaturated carbonyl compounds have powerful GSH depletion action but not much is known about their undesirable side effects. On the other hand, buthionine-SR-sulfoximine (BSO) is a powerful depleting agent with very high efficacy and very few if any harmful side effects. It can also cause depletion in almost all tissues where GSH is synthesized.

2.12 L-Buthionine –SR-Sulfoximine (BSO)

BSO is a synthetic amino acid that depletes glutathione irreversibly inhibiting gamma-glutamylcysteine synthetase, the rate limiting enzyme in glutathione synthesis. BSO competes with glutamate for the same binding site. As such, the rate and extent of BSO inhibition depends on the concentration of glutamate. Adding 10 μ M BSO in presence of 5 mM glutamate and 10 mM ATP, led to 100% inhibition of γ -glutamylcysteine synthetase in rat kidney in 10 minutes (97). Buthionine sulfoximine inhibits GSH biosynthesis by effectively inhibiting γ -glutamylcysteine synthetase, the rate limiting enzyme in GSH biosynthesis (68).

2.13 Routes of BSO Administration

BSO can be administered in various ways to bring about GSH depletion. The most commonly used routes are oral and intraperitoneal. Both methods can produce very high level of GSH depletion. However, both methods also cause wide spread GSH depletion with some organs such as the liver affected more than others. Other organs such as the brain are only slightly affected. This global depletion of GSH which is not confined to one site can cause alterations in the overall homeostasis of the animal. Thus, both routes can not be used to produce depletion at one site alone.

Convection enhanced delivery (CED) is a relatively new method of delivering drugs directly into the brain or the spinal cord. This method bypasses the BBB (blood brain barrier) ensuring maximum delivery of the drug to the brain. The BBB is a membranous structure composed of endothelial cells that are very tightly packed in brain capillaries. This arrangement restricts the passage of substances from the blood to the brain much more than endothelial cells in capillaries elsewhere in the body. By this method, high local concentrations of the drug can be achieved without affecting the overall metabolism of the animal. Furthermore the procedure has been shown to be very safe in animals and humans as well (98).

2.14 BSO in Human Studies

Two human ovarian cancer cell lines (KK and MH) from the ascites of patients who did not respond to the anticancer agent cisplatin (CDDP)-based combination chemotherapy were found to contain higher than normal levels of glutathione. These cells were resistant to the drug CDDP in a manner corresponding to the level of intracellular glutathione. Preincubating these cells with BSO resulted in reduction of cellular glutathione to 9-25% of initial values. Incubating the cells with 10 μ M BSO, the cells were sensitized to CDDP and its analogues showing a decrease of 79-38% in the IC₅₀ (a measure of drug effectiveness) values (99). In another study, the effect of reducing glutathione using BSO on the cytotoxicity of cisplatin, carboplatin and radiation in human stomach cancer cell line (SNU-1) and ovarian cancer cell line (OVCAR-3) was analyzed. It was found that BSO effectively depleted the

intracellular glutathione concentration and enhanced the effect of both the drugs and radiation (100).

BSO has also been used to deplete glutathione in humans for enhancing the effect of cytotoxic drugs. In a phase 1 clinical trial, patients were given BSO in two cycles consisting of BSO alone intravenously every 12 hours for six doses and 1 week later the same BSO as cycle one with melphalan (LIPAM) 15 mg/m² intravenous one hour after the fifth dose. Doses of BSO were escalated from 1.5 to 17g/m². Using this regime, the only toxicity that was attributable to BSO was grade I or II nausea/vomiting in half of the patients. When glutathione content in mononuclear cells (PMN) and tumor tissues were measured at intervals following BSO administration, it was found that GSH content decreased over 36 to 72 hours reaching its lowest levels on day 3 when PMN glutathione levels were approximately 10% of control at BSO doses of 7.5 g/ m². Also at BSO doses of 13g/m² or more tumor GSH was reduced to about 20% of starting values on the third day in 5 out of 7 patients. The study also found that total-body clearance (CLt) and volume of distribution at a steady state (Vss) was dose dependent for both R- and S-BSO. The harmonic half lives for R-BSO and S-BSO were 1.39 hours and 1.89 hours respectively. The biomedically appropriate dose of BSO was found to be 13g/m² (101).

BSO is also used in a phase II clinical trial at McGill Centre for Translational Research in Cancer to measure the activity of intravenous melphalan (L-PAM) with BSO in patients with ovarian cancer refractory to taxol combination. In the trial patients will receive a continuous infusion of BSO for 48 hours. Melphalan is then administered and BSO infusion continued for another 24 hours. This is repeated every 3-4 weeks while the tumor is followed up using tumor measurements and tumor markers to evaluate the response to therapy. The study is ongoing and some promising responses have been observed in the patients studied so far (A Phase II Trial of IV-L-PAM and BSO in Patients with Relapsed or Refractory Ovarian Cancer: www.mctrc.org/en/rp/studies/bso.htm

3. Materials and Methods

Cell Culture Experiment

3.1 Tissue Culture

The C6 glioma cell line was obtained from the American Type Culture Collection. This line was produced by in random-bred Wistar-Furth rats by exposure to N,N – nitrosomethylurea (102). The cells are stored in the laboratory of Anatomy and Cell Biology in liquid nitrogen from where they were taken out and thawed by warming for 30 seconds in a water bath at 37°C while shaking moderately. The cells were then suspended in Dulbecco's modified eagle's medium (DMEM) with 15% heat-inactivated fetal bovine serum (FBS) and 0.2 mM glutamine, 50 µg/ml neomycin and 100 mg/l streptomycin and cultured on 100 mm Falcon tissue culture plates. Five $\times 10^5$ C6 glioma cells were planted into each culture plate. The cultures were maintained for 48 hours in an incubator under an atmosphere of 5% CO₂ and 95% air at temperature of 37 °C.

Cultures were examined daily for the morphology and color of the medium as well as density of the cells. Three days following planting of cells the culture medium was replaced by a new growth medium to ensure that cells receive adequate nutrition. During the first 48 hours the C6 cells go through a lag phase during which there is minimal or no growth before going into the exponential growth phase when they have a high metabolic rate as well as increased proliferation rate. The cells finally enter a stationary phase when the cells become confluent on the culture plates and there is no room for further expansion (103).

3.2 Addition of BSO

On the third day after cell planting, BSO was added to the growth medium at concentrations of 1, 10, 100 and 1000 µM respectively. No BSO was added to the control group. There were a total of 10 cell culture plates with two per group. The BSO was reconstituted by dissolving in sterile water the powder form of BSO (C₈H₁₈N₂O₃S) stored at 2 – 8 °C. First a stock of 10 mM BSO is made and further concentrations were made using the formula $C_1V_1 = C_2V_2$ to obtain the required concentration. The working concentration was then added to the culture plates which

were then returned to the incubator and allowed to stand for 24 hours before measuring GSH concentration.

3.3 Cell Harvesting and GSH Measurement

GSH concentration of the cells was measured using the monochlorobimane method as outlined in Kamencic et al (104). First the cell cultures were inspected under the microscope to make sure they were healthy and growing well. The growth medium was then exchanged with a serum free medium (without FBS) and 100 μ M of monochlorobimane added. After 30 minutes the cells were washed with phosphate buffered saline (PBS) twice. Ethylene diamine tetraacetic acid (EDTA) was used to detach the cells from the culture plates. They were then washed with a Puck's solution before adding harvesting medium. Cultures were then returned to the incubator and left for 30 minutes under the same conditions to allow them to detach from the culture plates and became rounded. Manual washing of the cells using a pipette was then done to ensure all cells were detached before being transferred to a culture tube. They were then spun for 10 minutes at 1000 rpm. The supernatant was discarded and replaced with Hank's solution. The cells were then sonicated for 5 s thrice. This was followed by centrifugation at 13000 g for 10 minutes. The supernatant was aspirated and its glutathione content of the samples and standards were measured using a fluorimeter (Fluoroskan Ascent[®] from Thermo Scientific) at the City Hospital Saskatoon.

3.4 C6 Experimental Groups

To study the effect of radiotherapy on the C6 glioma cells, the cells were planted in 100 mm plates as described above. They were divided into three groups with 12 culture plates in each group. The first group acted as a control group. The second group received radiotherapy only while the third group received BSO and radiotherapy. BSO was added as described above at a concentration of 100 μ M solution 24 hours prior to radiotherapy. Each group was further divided into three subgroups that were harvested at 2, 24 and 48 hours following treatment. Each subgroup consisted of 4 culture plates. The cultures were maintained at the specified

conditions above at the Department of Anatomy and Cell Biology Central Tissue Culture Laboratory.

3.5 Transport of Cell Cultures

Transportation of the culture plates from the Department of Anatomy and Cell Biology to the Cancer Clinic was done using modulator chambers. These were humidified by placing a water filled Petri dish on the bottom of the incubator chamber. The cell culture plates were then placed in the chamber and the chamber continuously flushed with 5% CO₂ in air for 5 minutes (Fig 3.1). This ensured that cultures were maintained within the same pH during the transport time which is approximately about 7- 10 minutes.



Fig 3.1: Flushing the transport chambers with 5% carbon dioxide.

3.6 Irradiation of C6 Cell Cultures

Radiation therapy was done at the Department of Radiotherapy of the Saskatoon Cancer Clinic. A single dose of 10 Gy was administered to each cell culture plate (Fig 3.2). Irradiation was of photon type at 150 KeV. There is about $\pm 5\%$ variation of radiation dose throughout the irradiation field. All 36 plates of C6 glioma cell cultures were irradiated.



Fig 3.2: C6 culture plates being placed for irradiation with 10 Gy.

3.7 Cell Counting

Cells were isolated from the tissue culture dishes using harvesting medium as described above and then counted using a hemocytometer at 2, 24 and 48 hours respectively following radiotherapy. Nigrosin-containing solution was added in a ratio of 1:4 to the cell suspension (Nigrosin 0.03% concentration in a balanced salt solution is used as a dye in this method. Live cells do not take up this dye while dead cells, due to loss of the semipermeable membrane take up the dye and stain black). The two were gently mixed and allowed to stand for 15 minutes. A small amount was transferred to one side of the hemocytometer while the cover slip was in place. This was done by carefully touching the edge of the cover slip with the tip of the pipette and allowing each chamber to fill by capillary action. Starting with one chamber of the hemocytometer cells were counted in the 1 mm centre square and four 1 mm corner squares using a 100X light microscope magnification. A separate count of viable and dead cells was kept. Cells on top and left touching middle line were included while those touching middle line at bottom and right were excluded from the count. Four corner squares in one chamber were counted and the average was calculated. Each large square of the cover in place represents a volume of 0.1 mm^3 or 10^{-4} cm^3 . Since 1 cm^3 is approximately equal to 1 ml, the total number of cells per ml was determined using the formula:

$$\text{Cells/ml} = \text{average cell count per square} \times \text{dilution factor} \times 10^4$$

Total cells were then calculated by multiplying the cells/ml by the original volume of cells from which the sample was taken.

Animal Experiment

To study the effect of BSO on the animal model glioma adult male Wistar rats weighing 250-300 gm were used for the experiments and were obtained from Charles River, St Constant, Quebec. In the first experiment, the effect of BSO on normal brain and tumor tissue was evaluated. In the second experiment the effect of RT and BSO in treating the tumor harboring rats was studied. For both experiments, the animals had intracranial implantation of the C6 glioma cells. Prior to implantation, C6 cells were cultured and harvested in the same way described above. They were then resuspended in Hanks solution and constituted to a concentration of 10×10^4 cells/ μ l of solution. They were then ready to be implanted into the brain.

3.8 Intracranial Implantation

The Animal Ethics Committee of University of Saskatchewan approved this experimental protocol. Rats were anesthetized with halothane using an anesthetic machine before positioning on a stereotaxic frame (Fig. 3.3). The hair was shaved and the head cleaned with iodine and alcohol. A skin incision of about 1.5 cm in length was made in the mid-line starting behind the eyes and extending backwards. A burr hole was made about 2.5 mm behind the coronal suture and about 2.5 mm to the right of the midline using a small drill MiniMite[®] from Dremel. C6 cells were then implanted using a 27 G needle attached to a Hamilton syringe. The syringe was attached to the stereotaxic frame and using a micropump, 10 μ l of aliquot containing 100,000 cells were injected to a depth of 4mm at a rate of 2 μ l per minute. The burr hole was then sealed with bone wax and the incision closed. Subcutaneous buprenorphine (Buprenex) was administered to relieve pain. This was diluted with normal saline in a ration of 1:5 and given at a dose of 0.05 mg/kg. After recovering from anesthesia the rats were returned to their housing facility. They were followed up for a period of 13 days during which they were monitored for any symptoms and were weighed 3 times weekly.



Fig. 3.3: A rat placed in a stereotaxic frame under general anaesthesia prior to implantation of C6 glioma cells.

3.9 MRI of Tumor Harboring Rats

On the 13th day following implantation MRI was conducted on all the rats. This was done at the Department of Medical Imaging, Royal University Hospital, Saskatoon using a Siemens Symphony 1.5 tesla with Syngo 2002B software. The images are Turbo Spin Echo T2 weighted. The rats were sedated using a combination of ketamine and xylazine prior to imaging.

3.10 Glutathione Estimation

On the 14th day after implantation a total of 6 rats were injected with BSO using the same set up for C6 implantation. Through the previous burr hole 4 μ L of 100 μ M BSO was injected to a depth of 3 mm into the tumor tissue using a micropump at a rate of 2 μ L per minute. The burr hole was then sealed using bone

wax. BSO was also injected in to the brain of 6 healthy rats at a site corresponding to the site of tumor in the experimental rats. This acted as the control group. Two rats each from both experimental and control groups were then sacrificed at 0, 2, and 4 hours. The tumor tissues was harvested and immediately immersed in liquid nitrogen prior to glutathione estimation. The glutathione content of both tumor tissue and healthy brain samples was then measured using high-performance liquid chromatography (HPLC) according to the procedure outlined in Kamencic et al (105).

3.11 Radiotherapy for Rats

According to the type of treatment they received, the animals were divided into six groups. The first group received BSO and radiotherapy, the second group normal saline and radiotherapy, the third group radiotherapy alone, the fourth group BSO alone, the fifth group normal saline alone and the sixth group received no treatment and was allowed to run the natural course of the disease. There were a total of 30 rats. Radiotherapy was administered at the Cancer Clinic at the Royal University Hospital, Saskatoon. A single dose of 10 Gray was given using a 16 MeV machine. The rats were again sedated using a combination of ketamine and xylazine prior to radiotherapy.

3.12 End of Experiment

Following treatment, animals were returned to their housing facility and followed up. Those that became symptomatic were sacrificed when symptoms became severe. When no treatment is administered, symptoms of increased intracranial pressure usually appear after 18 to 25 days following implantation and consist of slowing down of activities, sluggishness and later contralateral hemiparesis. There is decreased food intake with loss of weight, impaired grooming and later circling and seizures. Without intervention, the rats go on to develop stupor followed by the demise of the animal. Rats were sacrificed once they develop contralateral paralysis, circling or seizures. The long term survivors were sacrificed at the end of the experiment which was 100 days post implantation of C6 cells. Sacrificing of rats was done by trans-aortic perfusion with paraformaldehyde under general anaesthesia.

3.13 Fixation and H & E Staining of Brain Tissue

The whole brain was then removed from the cranium and sectioned into three parts before embedding in paraffin using a Tissue-Tek[®] VIP machine from Miles Scientific. The embedded samples were sectioned at 10 µm thickness and mounted on Colorfrost[™] Plus from Erie Scientific. They were then stained using Hematoxylin and Eosin (H&E). Hematoxylin is a base and stains acidic cellular components (mainly DNA and RNA) dark blue. Eosin is an acid and stains basic components which include most of the cytoplasmic components of the cell pink.

The protocol used for staining with H & E involves the passage of paraffin sections through a series of solutions for specified times. These solutions include xylene, absolute alcohol, 95% ethanol, 70% ethanol, and tap water respectively before rinsing in distilled water. The slides are then immersed in Ehrlich's Hematoxylin for 10 minutes and rinsed in tap water. They are then dipped 4 times in acid alcohol and washed in tap water before immersing in saturated lithium carbonate (1.36 g/100 ml). Subsequently, they are rinsed in running tap water followed by distilled water before being immersed in Eosin Y solution for 1 minute. They are then removed, rinsed again and examined under the microscope to make sure that appropriate amount of staining is achieved. The staining was adequate and the slides were then dipped for specific times in 95% ethanol, absolute alcohol, absolute alcohol/xylene and finally xylene. Finally, in a fume hood Cytoseal was applied to cover slips and then mounted on to the slides. The slides were then allowed to dry and were then ready for histological examination.

4. Results

4.1 The Effect of BSO on C6 Glioma Cell Culture

The addition of BSO to cell cultures led to the reduction of GSH synthesis. There was a dose-dependent reduction in GSH levels to a certain concentration. The maximal reduction occurred at a concentration of 100 μM BSO leading to reduction of GSH of more than 80% of baseline values. There was no further reduction in GSH concentration at a higher concentration of 1000 μM BSO (Fig 4.1). (n = 10, 2 per group).

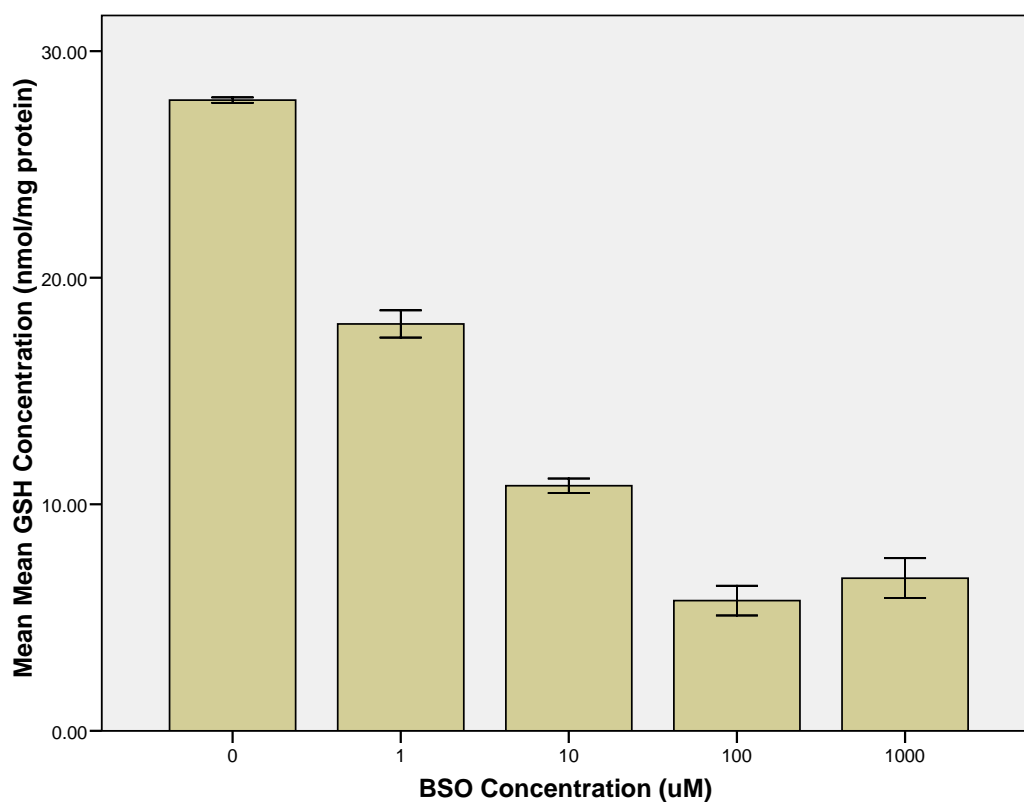


Fig 4.1: GSH levels of C6 Glioma cells following 24 hour exposure to BSO. Error bars represent standard error of the mean.

4.2 Effect of Radiation Therapy on the Growth of C6 Glioma Cells

After 2 hours from the time of RT, the control group had a mean cell count of $70.1 \pm 2.13 \times 10^4$. Following treatment with RT (10 Gy) either alone or in combination with BSO the mean cell count was found to be $68.43 \pm 5.1 \times 10^4$ and $68.81 \pm 4.41 \times 10^4$ respectively, with the errors being standard deviations (Fig 4.2).

In the next 24 hours the mean cell count of the control group increased to $106.87 \pm 8.23 \times 10^4$. Those that received RT had a similar growth rate with a mean cell count of $106.12 \pm 11.09 \times 10^4$ while the group that received RT and BSO had a slower growth rate with a mean cell count of $92.37 \pm 9.78 \times 10^4$ (Fig 4.2).

At 48 hours the cells in the control group had greatly multiplied to a mean cell count of $190.93 \pm 5.48 \times 10^4$ while both the groups that received either RT alone or in combination with BSO had much slower growth rate with a mean cell count of $137.68 \pm 9.56 \times 10^4$ and $107.93 \pm 9.2 \times 10^4$ respectively (n = 4 in all groups) (Fig 4.2).

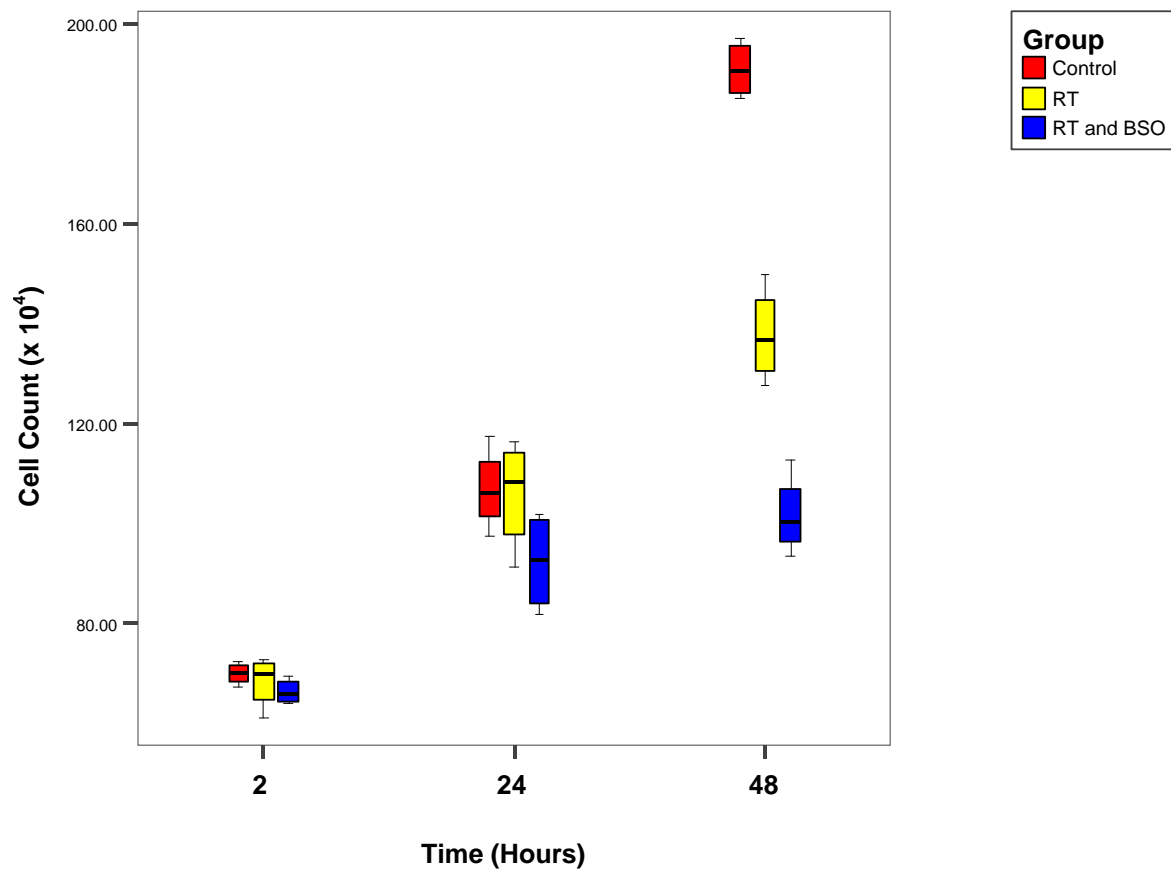


Fig 4.2: Effect of radiation therapy (10 Gy) on C6 cell growth over 48 hour period.

4.3 MRI of Rats Following Implantation of C6 Cells

All 30 rats were imaged on the 13th day post implantation. MRI confirmed the presence of tumor in all the rats that had been implanted with C6 glioma cells (e.g. Fig 4.3 & 4.4).

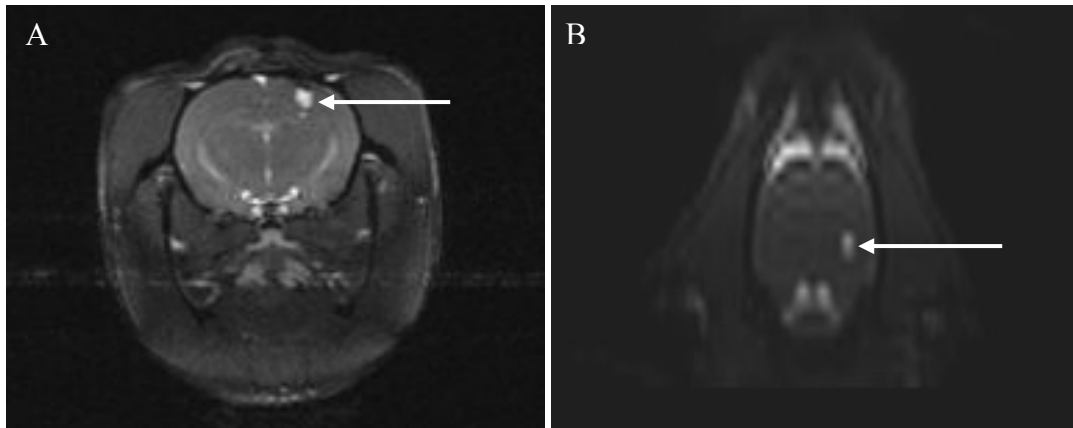


Fig. 4.3: Coronal (A) and axial (B) T-2 weighted MRI scans showing the tumor in the brain of a Wistar rat 13 days post implantation of C6 cells.

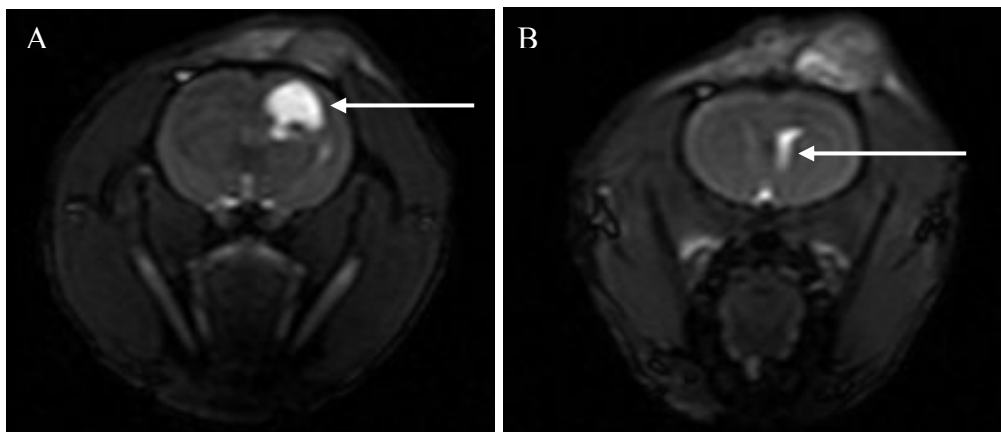


Fig 4.4: Coronal T-2 weighted MRI scans showing the tumor (A) and dilated lateral ventricle (B) in the brain of Wistar rat 18 days post implantation of C6 cells.

4.4 Effect of BSO on Normal Brain and Brain Tumor

The glutathione concentration of normal brain tissue was found to be 31.65 nmole/mg protein (SD = 0.25) while that of tumor tissue was found to be 44.18nmole/mg protein (SD = 2.0). Injection of BSO into the brain and tumor tissue resulted in decrease of GSH concentration in both the normal brain and tumor tissue. The effect was maximal at 2 hours with normal brain GSH reduced to 28.13 nmole/mg protein (SD = 1.8) while the glutathione content of the tumor tissue was reduced to 11.37 nmol/mg protein (SD = 4.0) (Fig 4.5).

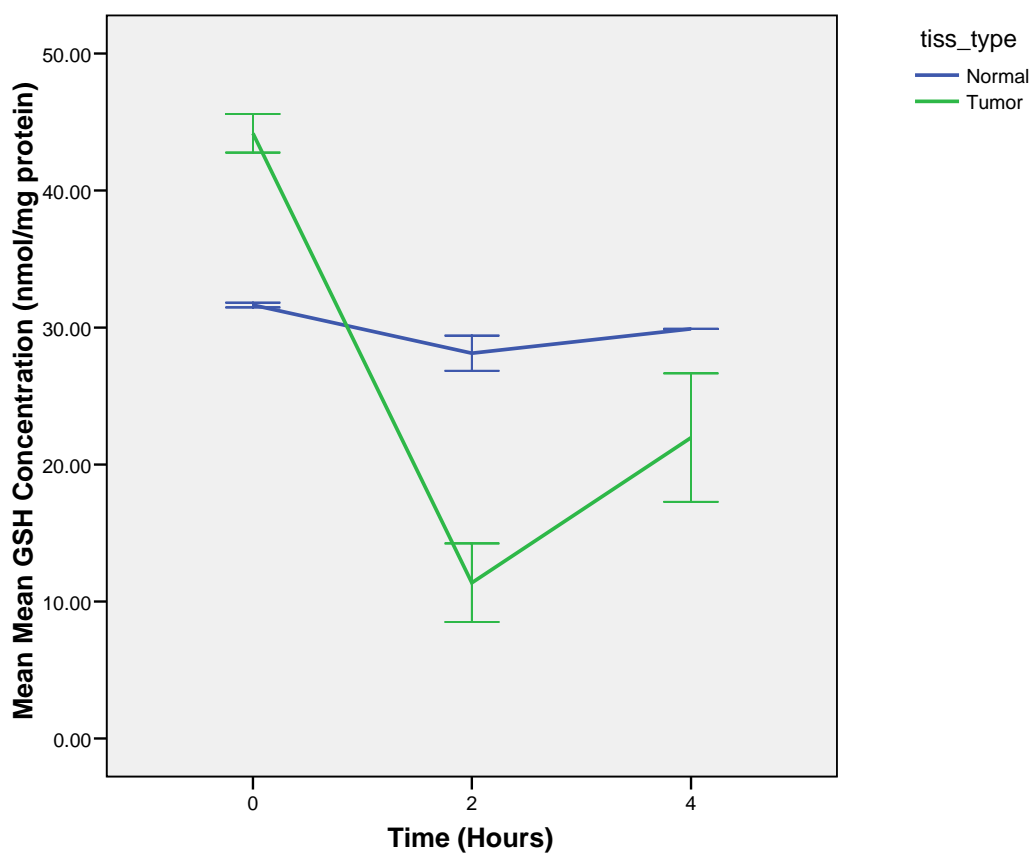


Fig 4.5: Time-dependent effect of BSO on normal brain and tumor tissue GSH. Error bars are one standard error of the mean

4.5 Survival of Rats Following Treatment

Rats that received no form of treatment ($n = 4$) had a median survival time of 16 days. Those that received BSO ($n = 4$) and normal saline ($n = 4$) as the only form of treatment had a median survival time of 18 days and 22 days respectively. The rats that received radiotherapy as the only form of treatment ($n = 6$) or a combination of normal saline and RT ($n = 6$) had a longer median survival time of 40 days for RT alone and 35 days for normal saline and RT alone respectively. Rats that had BSO and RT as a form of treatment had 4 out of 6 rats surviving at the end of 100 days compared to one surviving rat out of each of the 6 rats that received either RT alone or in combination with normal saline (Fig. 4.6).

Using Cox regression analysis the effect of radiotherapy and BSO on the survival of rats was analyzed. The odds of survival for those that had radiotherapy is 12.8 (95% confidence interval 3.7 to 44.3) times greater compared to those that had no radiotherapy in their treatment ($p < 0.001$) (Fig. 4.7). Rats that had BSO incorporated in their treatments had an odds of survival of 2.88 (95% confidence interval 1.09 to 7.64) times greater compared to those that had no BSO at all ($p < 0.05$). The 2 factors combined are significant predictors of survival ($p < 0.001$) (Fig. 4.8).

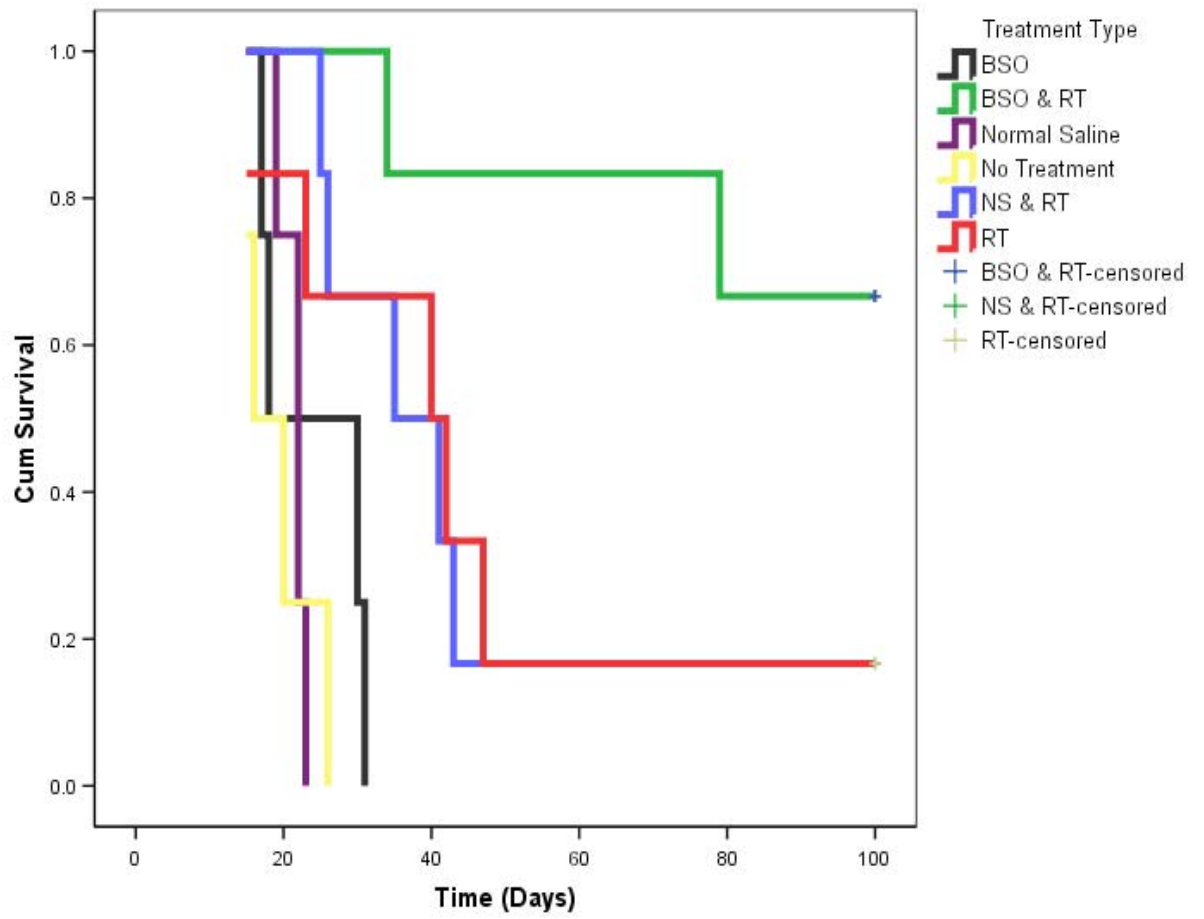


Fig. 4.6: Survival curve of animals following different forms of treatment.

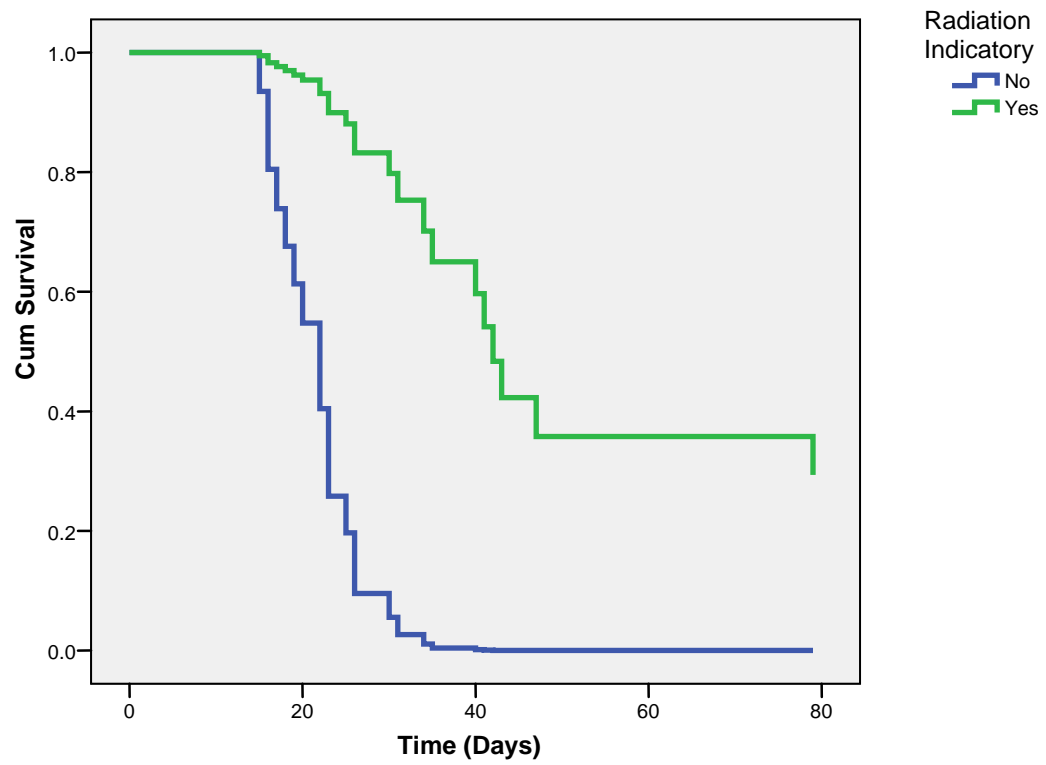


Fig. 4.7: Survival functions for the effect of radiotherapy on survival of rats.

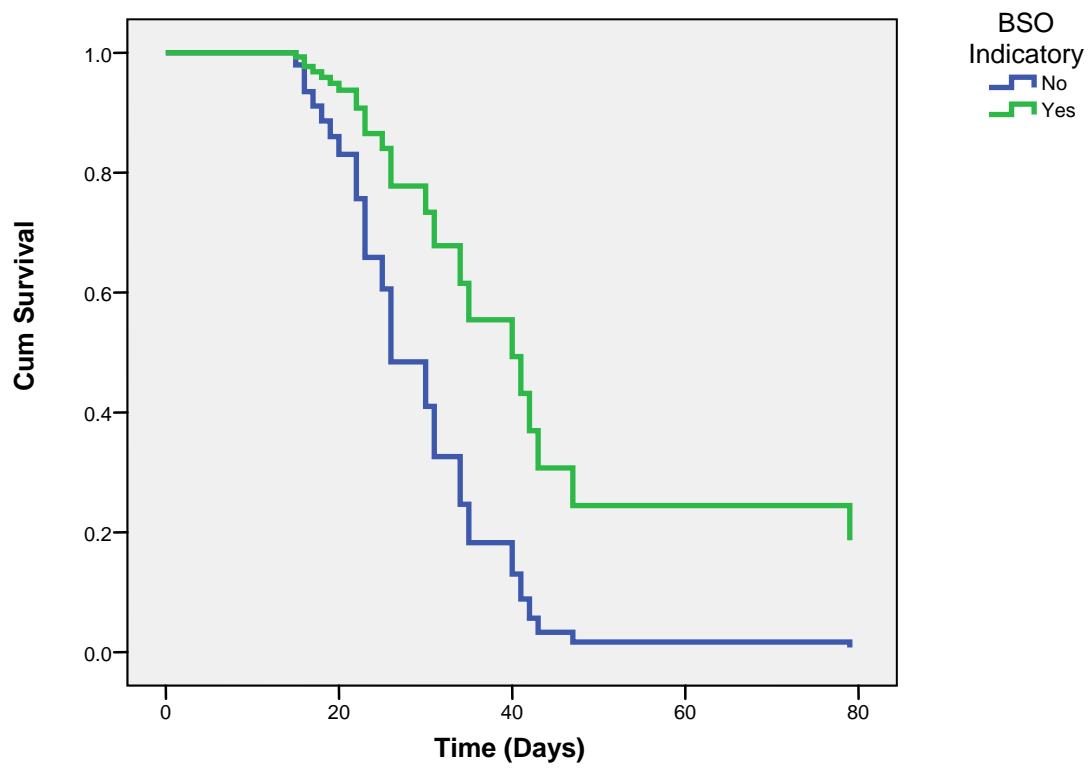


Fig. 4.8: Survival functions for the effect of BSO on survival of rats.

4.6 Histology

All 24 rats that had become symptomatic were euthanized. At the end of 100 days post implantation of C6 cells, 6 rats were still surviving. These were also euthanized at this time. Histological analysis of the brains of all 30 rats was carried out to look for the presence of tumor cells. The C6 cells are injected into the cerebral cortex at a depth of 4 mm. At 13 days post implantation, the tumor is fairly large and deeply situated within the cortex (c) (Fig. 4.10). At this stage the tumor can be easily visualized on MRI scans. Its effects on the surrounding brain can also be seen on the histological slide with slight dilatation of the ipsilateral lateral ventricle (v). Parts of the tumor have infiltrated into the deeper structures across the corpus callosum (cc) and into the thalamus (t). At this stage however, no gross changes are visible on the contralateral side.

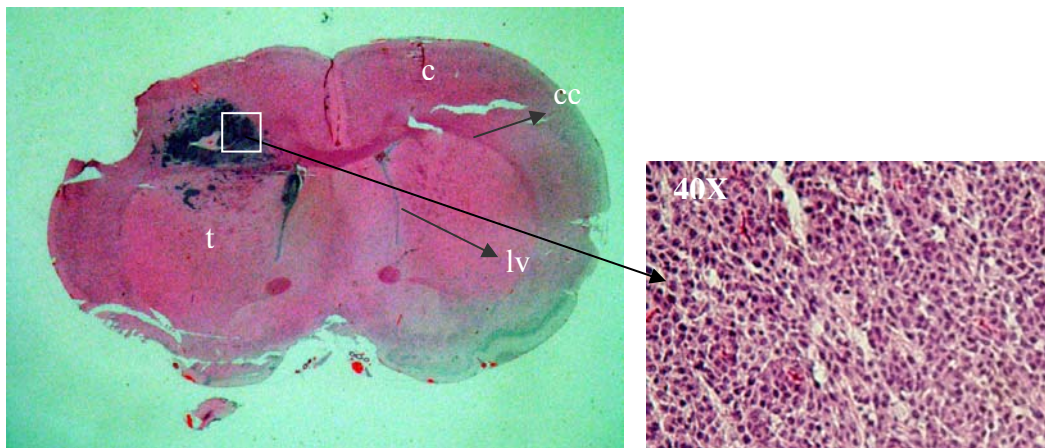


Fig. 4.9: Section of a rat's brain (H&E stain) at 13 days post implantation of C6 cells. The dark staining tumor can be seen in the right cortex with some projections into the thalamus. A magnification of the tumor cells is shown on the right.

At 26 days post implantation, the tumors are very large with massive infiltration into the brain tissue. Most of the ipsilateral cerebral cortex is destroyed by tumor tissue with crossing of the midline into the contralateral cortex. The ventricles are grossly dilated and there is substantial invasion of the thalamus by tumor tissue. At this stage the rat is usually very symptomatic and had to be euthanized.

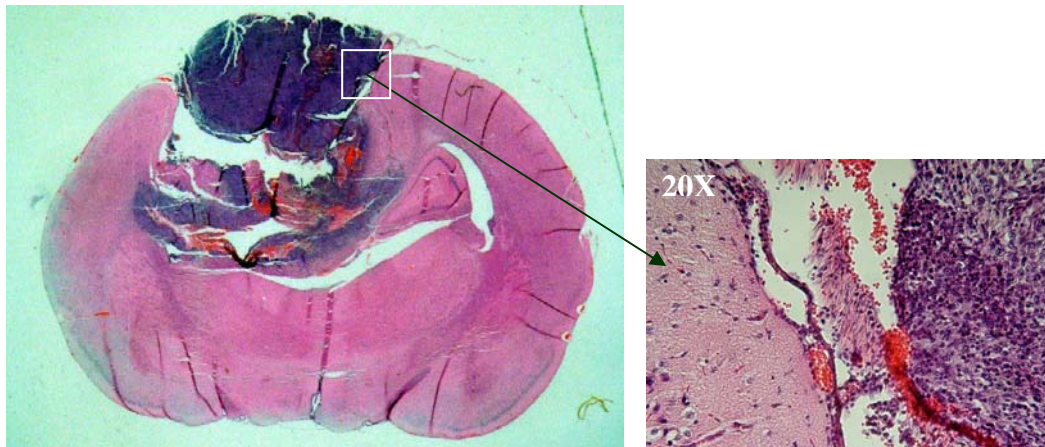


Fig 4.10: H & E stain of a rat's brain 26 days post implantation showing the extent of tumor growth. The region in the box is magnified on the right depicting the tumor cells.

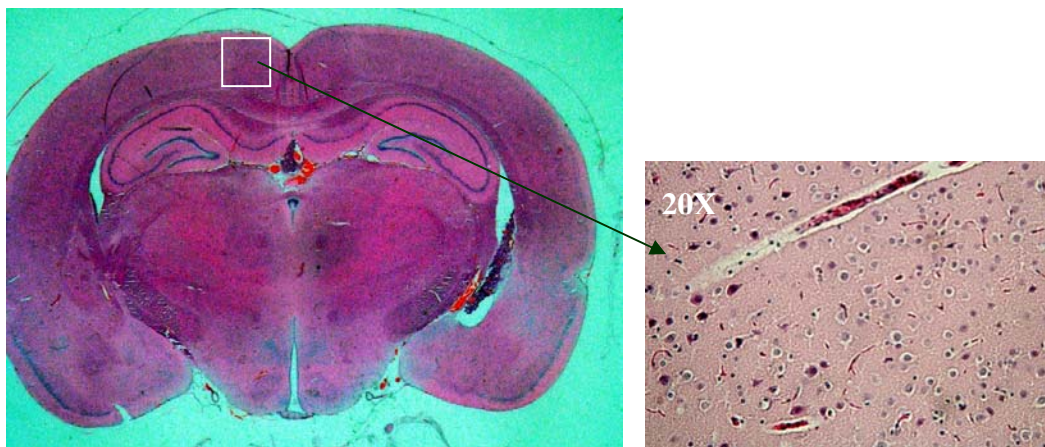


Fig 4.11: H & E stain of a rat's brain implanted with C6 glioma cells that was treated with RT and BSO at 100 days following treatment. To the right is a magnification of the region of tumor cell implantation. No tumor cells could be detected.

5. Discussion

5.1 Choice of Animal Tumor Model

C6 cell transplanted in Wistar and Sprague-Dawley rats were found to have characteristics closer to natural glioblastoma in humans than tumor grown in other strains of rats (106). When injected intracranially at a total number of 100,000 cells, there was a 100% take with the cells growing in a predictable fashion and the tumor was consistently lethal in non-radiated animals (107). The tumor's glial nature, its stability in tissue culture and its consistent ability to grow intracranially in the Wistar rat made this a good model for this study. There was a 100% uptake of tumor in our animal models as confirmed by MRI.

5.2 Choice of Radiosensitizing Agent

Aerobic metabolism in organisms leads to oxygen radical stress as a byproduct. This results in the formation of highly active intermediates, namely hydrogen peroxide and superoxide that promote oxygen radical production which leads to cellular damage and increased production of oxygen free radicals (41). Being the major endogenous soluble antioxidant in mammalian cells, GSH provides protection against this oxidative stress (42)-(43). The various functions of GSH have been previously outlined.

Different methods have been used to lower GSH levels. However, each method has its own drawbacks mainly in the form of unwanted side effects which can be quite serious in some instances ranging from nephrotoxicity to convulsions and death with certain compounds. BSO was found to have no side effects when administered to animals. In humans, the only side effects observed were nausea and vomiting and a slight decrease in white cell count (101). No side effects were observed in the animals that were given BSO (92, 94, 95).

5.3 Effect of BSO on Cell Culture

The reduction in GSH concentration correlates with findings in other studies where BSO was used to reduce GSH levels. Beyond 100 μ M solution of BSO no further dose dependent GSH reduction was seen. This is due to the fact that BSO acts

as a competitive inhibitor of γ -glutamyl-cysteine synthase and at this concentration saturation occurs.

5.4 Effect of RT and BSO on C6 Cell Culture

The effect of radiotherapy is achieved mainly by causing the tumor cells to lose their ability to reproduce and thus cause a gradual reduction in tumor size. Thus as time goes on, more and more cells are lost as they try to divide and multiply. In certain cases, the aim is to cause tumor ablation altogether using higher doses. The effect on tumor growth is brought about by damage to the cell DNA. This is induced either by primary photons or charged particles from the radiation source causing direct damage to the DNA or by the formation of free radicals and other oxidants due to interactions within the cell. These free radicals and oxidants in turn cause DNA damage and are toxic to the cell.

After 2 hours following radiotherapy either alone or in combination with BSO, there was no significant difference in the cell count of all three groups. This shows that there was no direct killing effect of radiation such as seen with very high doses of irradiation. After 24 hours, there was some difference in cell count between the groups. While the group that received both RT and BSO showed the smallest increase in cell proliferation, the effect was minimal and not statistically significant.

After 48 hours however the difference in cell proliferation was more marked. While the control group continued to grow in an exponential fashion, both the two other groups were growing at a much slower rate. Cells that received RT alone showed a significant difference ($p < 0.05$) in cell number compared to the control group while those that received a combination of RT and BSO showed an even greater slowing down in cell proliferation ($p < 0.001$). BSO in combination with RT was superior in arresting cell proliferation (and hence causing cell death) compared to RT alone with the difference being statistically significant ($p < 0.05$).

5.5 Route of BSO Administration to Animals

BSO can be administered in various ways to bring about GSH depletion. The most commonly used routes are oral and intraperitoneal. Both methods can produce marked reduction in the level of intracellular GSH. However, both methods also cause wide spread GSH depletion with some organs such as the liver being affected more than others. Other organs such as the brain are only slightly affected. This widespread depletion of GSH which is not confined to one site can cause alterations in the overall homeostasis of the animal. These routes are thus unsuitable to bring about GSH depletion in the brain alone.

In Convection-enhanced delivery (CED) a drug can be delivered locally into the brain using a positive pressure gradient or bulk flow. It also allows the blood brain barrier (BBB) to be bypassed ensuring maximum delivery of the drug to the brain. By this method high local concentrations of a drug can be achieved without affecting the overall metabolism of the animal (98). The procedure has been shown to be very safe in rat tumor models (108). We thus preferred this method to administer BSO and induce GSH depletion in the brain tumors of the rats. The BSO administered was quite effective in reducing the GSH level in the tumor but had little effect in normal brain tissue. As previously cited BSO acts mainly on actively dividing cells where there is new production of glutathione. In normal brain, where there is little or no cell division taking place, the effect of BSO is very minimal.

5.6 Survival Outcomes and Statistical Analysis

A total of 30 rats were used for this experiment. Survival times were determined from the day of tumor implantation to the day of euthanasia. Euthanasia was done when symptoms which included weakness, tremors, loss of weight and circling became severe enough to prevent the animal from moving around and feeding. From our experience animals that are not sacrificed at this point usually died a day later and thus one day was added to all the animals that were euthanatized due to these symptoms. The Statistical Package for the Social Sciences (SPSS) program was used and survival data were analyzed using Kaplan-Meier test.

When rats that were allowed to run the natural course of the disease were compared to those that received BSO or normal saline as the only form of treatment, there was no statistically significant prolongation in the median survival time of any of the two groups ($p < 0.2$ & 0.5) respectively. This showed that neither BSO nor normal saline on their own had any effect in changing the course of the disease in the rats.

When compared with those that had no treatment, rats that received RT alone had a statistically significant ($p < 0.048$) increase in median survival time. The survival of rats that had a combination of normal saline and radiotherapy was the same as those that received radiotherapy alone ($p < 0.86$). Normal saline has no known radiosensitizing effect on malignant tumors. Thus the combination therapy using normal saline and radiotherapy had a similar outcome to that of radiotherapy alone.

The difference in survival times between rats that received a combination of BSO and RT and the group that received no treatment at all was highly significant ($p < 0.001$). When compared with animals that received RT alone there is a marked difference ($p < 0.059$). The effect of radiotherapy across all groups in which it was administered was analyzed using Cox regression. Those that received radiotherapy were about 12.8 times more likely to survive compared to those that did not receive radiotherapy either alone or in combination ($p < 0.001$). This is expected since radiotherapy is the main treatment modality for this type of tumor. For rats that received BSO compared to those that did not the survival probability was much less being 2.88 ($p < 0.05$). This shows that while BSO on its own had little impact on the outcome of the tumors, it did have some beneficial effect which could be attributed to its antioxidant properties as previously discussed. The combined effect of radiotherapy and BSO in predicting survival outcome was highly significant ($p < 0.001$).

Four rats out of 6 were still surviving at the end of 100 days among those that received a combination of BSO and radiotherapy compared to 1 rat each in the groups that received radiotherapy alone and a combination of radiotherapy and normal saline. It is not surprising to have a long term survivor among rats that received radiotherapy alone since cure of many cancers can be achieved using this form of treatment even though this form of cancer is not known to be curable by radiation alone. The combination of normal saline and radiotherapy had no additional effect on long term

survival compared to radiotherapy alone and this too is expected as normal saline is not a known radiosensitizer. However, the combination of BSO and radiotherapy as a form of treatment for this cancer is highly superior when compared to radiotherapy alone as shown by the median survival times of animals in both groups as well as the number of animals still surviving at the end of the study.

5.7 Conclusion

The role of BSO in enhancing the effect of chemotherapeutic drugs has already been established as evidenced by the ongoing phase II clinical trial using a combination of BSO and IV-L-PAM in the treatment of patients with relapsed or refractory ovarian cancer at the McGill Centre for Translational Research in Cancer (MCTRC). This study showed that the effect of radiotherapy in treating glioblastoma in an animal model can be significantly enhanced by using BSO as a radiosensitizing agent. It is possible that following further studies and evaluation, this form of combination treatment could play a significant role in the ongoing battle against this highly malignant form of brain tumor.

6. References

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